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REFUGIAL PATTERN OF *BROMUS ERECTUS* IN CENTRAL EUROPE BASED ON ISSR FINGERPRINTING

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We studied the thermophilous grass *Bromus erectus* in Central Europe to determine its pattern of population genetic structure and genetic diversity, using ISSR-PCR fingerprinting to analyze 200 individuals from 37 populations. We found three genetic groups with a clear geographic structure, based on a Bayesian approach. The first group occurred west and south of the Alps, the second east and north of the Alps, and the third was formed by four genetically depauperated populations in Germany. The populations from Germany formed a subset of the Bohemian-Moravian populations, with one private allele. Two differentiation centers, one in the Atlantic-Mediterranean and the second in the Pannonian-Balkan area, were recognized by species distribution modeling. The geographic distribution of the genetic groups coincides with the syntaxonomic split of the Festuco-Brometea class into the Festucetalia valesiaceae and Brometalia erecti orders. We found a statistically significant decrease in mean ISSR bands per individual from south to north, and to a lesser extent from the east to west. The former was explained by Holocene long-distance migrations from southern refugia, the latter by the difference in the gradient of anthropopression. We hypothesize a cryptic northern shelter of the species in Central Europe in the putative Moravian-Bohemian refugium.

Key words: Festuco-Brometea, general linear model, glacial refugia, Poaceae, phylogeography, species distribution modelling.

INTRODUCTION

Phylogeographic analyses of plant and animal species are aimed at finding genetic markers in population samples taken randomly across their geographical ranges. The null model assumes the lack of genetic differentiation of a species, that is, panmixia. Falsification of the null model implies the existence of an alternative model, which is in fact a phylogeographic hypothesis. In recent years the phylogeographic hypotheses have dealt mainly with the Holocene history of species representing various ecological and geographical groups, mainly in the context of southern refugia and putative "northern" glacial refugia (Nieto Feliner, 2011).

Our study dealt with the genetic structure of the thermophilous grass *Bromus erectus*, a component of xerothermic grasslands. In Central and Western Europe they form the Festuco-Brometea class comprised of two orders: Brometalia erecti and Festucetalia valesiacae (Mahn, 1986). The first order consists of Submediterranean and Subatlantic calcareous grassland of the Xerobromion and Mesobromion alliances and is thought to be genetically linked with a refugium of xeromorphic vegetation located west of the Alps (Medwecka-Kornaś and Kornaś, 1977). Most species of Brometalia erecti generally do not reach Central Europe but may be found as adventitious elements (e.g., *Bromus erectus* or *Onobrychis viciaefolia* in Poland).

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There are a few isolated areas in Central/Western Europe where xerothermic grasslands with a relatively high number of steppe species occur, as in some valleys in the Alps, the upper Rhine valley and Bohemia (Eijsink et al., 1978). In Poland they occur in the Małopolska Upland, Lublin Upland, and the valleys of the lower Vistula and Oder.

Patches of xerothermic grasslands in Central Europe occur mainly on calcareous and chalk outcrops, steep river banks, eroded escarpments and alluvial cones. In recent years they are prone to degradation as a result of the abandonment of traditional management practices in Poland and other countries in Europe (Hansson and Fogelfors, 2000; Kotańska et al., 2001). For this reasons they are under protection within the Natura 2000 European network. On the other hand, new patches of xerothermic grassland have developed in places that were formerly cultivated land, characterized by high species richness, including agricultural weeds (Karlik and Poschlod, 2011).

We used the ISSR method, applied in studies on phylogenetic, inter- and intrapopulation variation and in phylogeographic research; the primers can identify numerous polymorphic loci in a single PCR reaction (Dangi et al., 2004; Sutkowska et al., 2007; Ilnicki et al., 2011).

Our study was designed to verify three hypotheses. First, we expect a decline in the genetic richness of *Bromus erectus* along the south-north axis. The "leading edge" hypothesis predicts a decline of the population genetic diversity of thermophilous biota away from the glacial refugia (Hewitt, 1999). It relies on the classical view of the existence of southern glacial refugia for many thermophilous animal species or large-seeded trees (Bhgawat and Willis, 2008), and is supported by the continuous presence of pollen of such thermophilous trees as *Abies*, *Carpinus*, *Corylus*, *Ostrya* and *Ulmus* in peat deposits (Tzedakis et al., 2002). The second hypothesis deals with the east-west axis and concerns the syntaxonomic division of xerothermic grasslands into Central-Eastern European Festucetalia valesiacae and Western European Brometalia erecti. We expect the genetic differentiation of the grass species to follow the syntaxonomic division. If so, it could mean that the geographic split of the vegetation unit was brought about not only by recent climatic conditions but also by historical factors including the migration routes of thermophilous biota from the putative glacial refugia.

Specifically, we posit (the third hypothesis) that cryptic glacial refugia of the xerothermic grass species could also have existed in Central Europe. Recent molecular studies suggest that the mesophilous grass species *Festuca pratensis* and *Lolium perenne* (Fjellheim et al., 2006) and the thermophilous species *Stipa capilata* (Hensen et al., 2010) and *Eryngium campestre* (Bylebyl et al., 2008) could have survived the last glacial maximum (LGM) in nonglaciated areas of Central Europe.

The phylogeographic and syntaxonomic analyses were supported by the species distribution modeling (SDM). SDM techniques employ statistical or mechanistic approaches to assess the relationship between species distribution and potential determinants (Guissan and Zimmermann, 2000), and are increasingly being applied in palaeobiology as a complement to fossil and genetic evidence (Svenning et al., 2011). To reconstruct some putative refugial regions of thermophilous grass species in Central Europe in the LGM, we used a representative sample of occurrence data from its current range, current climate data and a palaeoclimate scenario.

MATERIALS AND METHODS

STUDY SPECIES

Bromus erectus Huds. (Upright Brome – Poaceae, subgen. Festucaria) is species native to Europe, Britain, Ireland and North Africa, introduced to the northeastern United States. It is a characteristic species of the Festuco-Brometea class, (Meso-) Bromion erecti alliance (Mahn, 1986). In its ecological profile it is similar to *Brachypodium pinnatum* (Reichhoff, 1980). It is restricted to dry, nutrient-poor xerothermic grassland, mainly on calcareous, chalk and gypsum substrate and also on chernozems, loess and brown soils. The grass has its ecological optimum in calcareous soils and is well suited to water shortage (Braun-Blanquet, 1928). It occurs on roadsides and other disturbed sites, as well as in pastures. Sometimes it penetrates dry pine forest Dorycnio-Pinetum on calcareous gravels or terraces of alpine rivers in the northern Alps (Ellenberg, 1982). In Poland it occurs naturally only in the southern part, and in other areas it occurs as a hemiagriophyte (Korniak, 2002). A polymerase chain reaction (PCR) study attributed its present distribution in Poland to a single post-glacial expansion (Sutkowska et al., 2002).

In Central/Eastern Europe *Bromus riparius* Rehm. is more often found and is held to be a Pontic element (Walter and Straka, 1970; Towpasz and Mitka, 2001). Both *B. riparius* and *B. erectus* belong to subgen. *Festucaria* and form sister species (Sutkowska and Mitka, 2008). They are an example of vicariance. In Central Europe xerothermic grasslands are distinguished in two orders within the Festuco-Brometea class: Brometalia erecti and Festucetalia valesiacae (Zoller, 1954; Medwecka-Kornaś, 1958; Krausch, 1961; Eijsink et

TABLE 1. Primers used in PCR, primer sequences, total number of reaction products generated by each primer, and mean number of PCR products per specimen

Primer	Primer sequence	Number of PCR products	Mean number of PCR products per specimen
ISSR1	$(TC)_8C$	69	7
ISSR2	$(AG)_8T$	60	6
ISSR3	$(GGGTG)_3$	79	9
ISSR4	$(ATG)_6$	69	7
ISSR6	$(AC)_8G$	78	8
ISSR7	$(AC)_8T$	57	7

al., 1978; Willems, 1982; see Fig. S3). In Brometalia erecti the very rare plant associations Gentiano-Koelerietum pyramidatae and Onobrychido-Brometum erecti occur. The second type of xerothermic grassland, of the Festucetalia valesiacae order, forms a subcontinental element and occurs in Central Europe as remnants of sub-continental Pontic-Pannonian xerothermic grasslands of Central/Eastern Europe. According to Klika (1954), European xerothermic vegetation forms independent units and its syntaxonomic division reflects not homology but analogy caused by microclimatologic, orographic and biotic influences. They develop on sites not directly used by man, for example on steep escarpments between fields, stream banks, or steep slopes of hills and gullies. A special type of site includes those that contain remnants of old fortress ramparts and slopes of tumuli dating back to the Bronze Age (Towpasz and Mitka, 2001).

Patches of xerothermic grassland are under strict protection within the Natura 2000 European network. In France, for instance, such protection covers Diantho gratianopolitani-Festucetum pallenti, Carici humilis-Anthyllidetum montanae and Coronillo vaginalis-Caricetum humilis. In Poland it covers Sisymbrio-Stipetum capillatae (Festuco-Stipion class), Inuletum ensifoliae, and Thalictro-Salvietum (both Cirsio-Brachypodion pinnati class).

Outcrossing *B. erectus* is pollinated by the wind. Its caryopses have a long awn which facilitates dispersal by animals (epizoochory). Flowering takes place between May and July, and fruit dispersal from June onwards. The chromosome number for the species is 2n=8x=28, although this may vary from 28 to 117 (Armstrong, 1981). The lower than expected number of chromosomes results from chromosome diminution, and the higher number represents euploids or aneuploids. In *B. erectus* the chromosome numbers vary not only between but also within individuals, resulting from mitotic disturbances (Joachimiak et al., 2001).

Bromus erectus has not been included in any breeding program and there is no registered or local variety cultivated, so the genetic structure of the grass is unaffected by human activity.

SAMPLING

Bromus erectus was sampled across Western, Central and Southern Europe in Austria, Croatia, the Czech Republic, France, Germany, Hungary, Italy, Poland, Romania and Slovenia (Fig. 1b, Tab. 2) during the 2010–2011 growing seasons. Leaf fragments were selected from individual plants distributed 3 m apart and then dried in silica gel.

DNA ISOLATION AND ISSR ANALYSES

The ISSR (inter-simple sequence repeats) method is based on highly polymorphic sequences of satellite DNA, consisting of a number of nucleotide sequences (microsatellites) tandemly repeated in thousands of copies. PCR reaction products are segments of DNA located between regions and include microsatellite sequences (Stepansky et al., 1999).

DNA was isolated from fully developed leaves showing no symptoms of damage from insects and mold. DNA was extracted with a Genomic Mini AX Plant (A and A Biotechnology). The primers used were 2–5 (15–18) nucleotide (ISSR) repeats. The primer sequences were taken from Stepansky et al. (1999) and are shown in Table 1. Amplification was carried out with a 25 µl reaction mixture: 2.5 µl 10-fold concentrated reaction buffer supplied by the Taq DNA polymerase manufacturer (Fermentas), 1.5 mM $MgCl_2$, 0.19 mM of each dNTP (Fermentas), 27 pmol primer, 100 ng template DNA and 1.4 U Taq polymerase. Reactions were run with a 2720 thermal cycler (Applied Biosystems). Annealing temperature was 44°C for primers ISSR2, ISSR4 and ISSR7, and 47°C for ISSR1, ISSR3, ISSR5 and ISSR6. Optimal conditions for the reaction were as follows: initial denaturation: 94°C – 5 min; 42 amplification cycles: denaturation 94°C – 59 s, annealing 44°C (47°C) – 59 s, polymerization 72°C – 59 s, final polymerization 72°C – 7 min. A negative control reaction without DNA template was included in each amplification. To verify the results, 50% of the samples were amplified twice. ISSR reproducibility tests (Bonin et al., 2004) included within-plate (n=12) and between-plate (n=9) replicates independently analyzed from the DNA extracts.

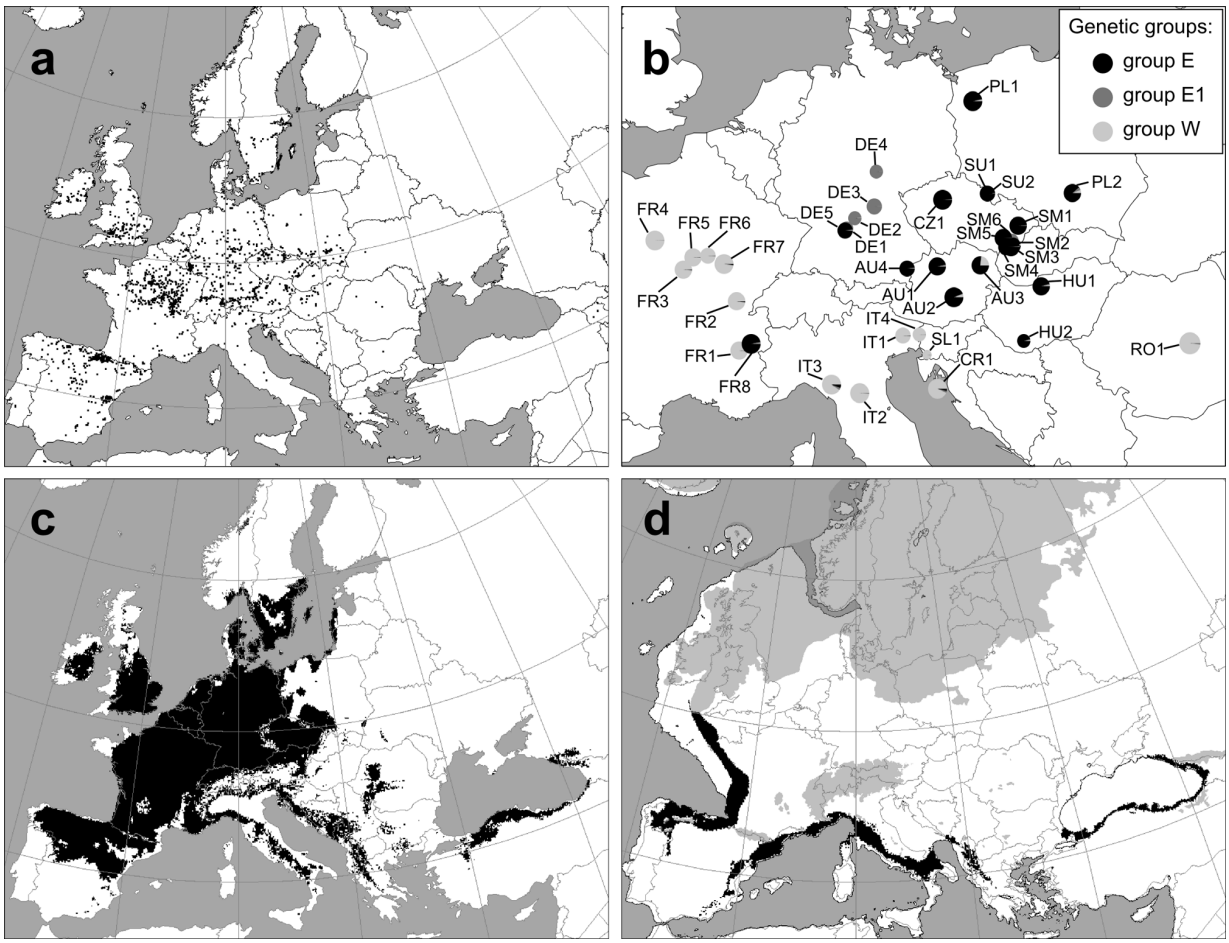

Fig. 1. Top row: (**a**) Locations of occurrence records of *Bromus erectus* used for calibration and evaluation of models (black dots), (**b**) Locations from which *B. erectus* samples were taken for molecular analyses, distinguished in three genetic groups: E, E1 (localities D1–D4) and W. Size of circles is proportional to mean number of bands per individual *FA* in the population. Bottom row: binary output of consensus model projections of potential species distribution (black area) onto (**c**) current climatic conditions and (**d**) LGM climate scenario. Consensus projections were derived by means of model predictions weighted by TSS scores. LGM ice sheet extent (lighter grey area) follows Ehlers and Gibbard (2004).

Products were subjected to electrophoresis in 1.5% agarose gel stained with ethidium bromide (50 µl/100 ml) at 100 V for ~1.5 h. Bands were observed and archived using an Imagemaster VDS (Pharmacia, Amersham) and Liscap Capture ver. 1.0.

Band pattern analysis employed GelScan ver. 1.45 (Kucharczyk TE). Using a calibration curve based on the band pattern of marker lengths (GeneRuler^{TM} 100 bp, Fermentas) we determined the molecular weight of the amplification products.

DATA ANALYSIS

The amplification products were scored as a presence/absence matrix of binary data. The genetic profile of the species was found using a Bayesian clustering algorithm for the use of dominant markers (Falush et al., 2007) implemented in STRUCTURE ver. 2.3.3 (Pritchard et al., 2000).The numbers of K = 2–10 groups were tested in ten replications per K. A burn-in period of 200,000, followed by 1 milion Markov chain Monte Carlo (MCMC) repetitions were used. An admixture model with uncorrelated allele frequencies was applied. The dominant ISSR data were analyzed by treating each class of genotypes as effectively being haploid alleles, according to the software documentation. Estimation of the optimal number of groups was based on the likelihood of partitions, estimates of posterior probability provided in STRUCTURE output examined as a function of increasing K (Pritchard et al., 2000), and ΔK values, estimating the change in the likelihood function with respect to K and estimated as an indicator of the most reliable clustering structure (Evanno et al., 2005). A model-based algorithm implemented in STRUCTURE was calculated on a megacomputer at the Bioportal of the

University of Oslo (www.bioportal.uio.no). Similarity between runs was estimated using the symmetric similarity coefficient (Nordborg et al., 2005) with Structure-sum R-script (Ehrich, 2006).

Analysis of molecular variance (AMOVA) was done and the fixation index F_{ST} was calculated using ARLEQUIN 3.5 (Excoffier and Lischer, 2010) with various groupings of populations into regions in order to determine which groupings give the highest discrimination of genetic variance among regions. Because the significance tests in AMOVA are based on nonparametric permutation procedures they are essentially assumption-free (Excoffier et al., 1992).

A matrix of bootstrapped (1,000 permutations) Nei and Li (1979) genetic distances was obtained with AFLPsurv ver. 1.0 (Vekemans et al., 2002). A neighbor-net diagram was made with SPLITSTREE 4.12 software (Huson and Bryant, 2006). Principal coordinate analysis (PCoA) using the same genetic distance measure was performed with NTSYSpc ver. 2.11 (Rohlf, 2002).

Population-level genetic variability was estimated using the rarity index *DW*, corresponding to frequency-down-weighted marker values per population (Schönswetter and Tribsch, 2005). R-script needed to calculate the *DW* index is available in AFLP-dat software (Ehrich, 2006). High *DW* values are expected in populations isolated long-term (Paun et al., 2008). The other measures of population genetic variability were the Shannon's diversity index I calculated with POPGENE ver. 1.31 (Yeh et al., 1999) and mean number of ISSR bands per individual *FA* in the population, with their standard deviations *FA(SD)*.

Kruskall-Wallis H and Wald-Wolfowitz Z tests for the statistical significance of mean differences among three genetic and between two geographic groups were performed with STATISTICA 10 (www.statsoft.com).

To find the relationships between geographic distribution and genetic index diversity *FA* we used the Poisson loglinear regression model with a quadratic term that assumes a Poisson distribution for the random components and uses the log link function for two explanatory variables: x (LATID) and y (LONG). The quality of the fitted model was checked using deviance-based tests: the F-test and AIC statistics (Agresti, 2007). The analysis was done using CANOCO for Windows (Ter Braak and Šmilauer, 2002).

RECONSTRUCTION OF THE POTENTIAL DISTRIBUTION OF *BROMUS ERECTUS* IN THE LGM

The source of occurrence data was the GBIF database (http://www.gbif.org) supplemented by literature sources (usually local floras) and numerous field studies we carried out over the past two years across Europe. The accuracy of the coordinates for data obtained from the GBIF database and literature was verified by comparing the location descriptions with data extracted from an electronic database (Hijmans et al., 1999). Finally, we identified and removed duplicate records by converting points on the grid at the same resolution as the predictors used. In total, we gathered 1552 occurrence records of *B. erectus* from Central and Western Europe for calibration and evaluation of the models (Supplementary material, Fig. S1a–d).

To reconstruct the potential distribution of *B. erectus* in the LGM we used a palaeoclimate scenario derived from the Community Climate System Model (CCSM, Collins et al., 2004). Bioclimate variables calculated on the basis of this model and downscaled to 2.5 arc-minute resolution were obtained from the WorldClim dataset (Hijmans et al., 2005, http://www.worldclim.org), together with present-day climate data at the same resolution. All the variables were tested for multi-colinearity by examining the cross-correlations among them (Pearson's r) based on the 1552 species occurrence records. Variables correlated at $r > 0.7$ were excluded from the models (Dormann et al. 2013), resulting in 5 variables representing temperature and precipitation: temperature annual range (*temp_ar*), mean temperature of warmest quarter (*temp_wq*), mean temperature of coldest quarter (*temp_cq*), precipitation seasonality (*prec_seas*) and precipitation of driest quarter (*prec_dq*). All environmental layers were limited to the same area between 10°W-50°E longitude and 35°N-72°N latitude, and covered the current distribution area and probably the LGM distribution of the studied species. Areas covered by ice sheet in the LGM (Ehlers and Gibbard, 2004) were excluded from the climatic layers of the CCSM paleoclimate scenario. Data set preparation, accuracy verification of occurrence records and reprojection of BIOMOD output maps to the ETRS89 reference system were done with GRASS GIS ver. 6.4 (http://grass.osgeo.org).

We used six different algorithms implemented in BIOMOD ver. 1.1-7 (Thuiller et al., 2009): two regression methods (GLM – generalized linear models; GAM – generalized additive models), two classification methods (FDA – flexible discriminant analysis; CTA – classification tree analysis) and two machine-learning methods (GBM – generalized boosting model; RF – random forest for classification and regression). To meet the minimum requirements of the algorithms used (Barbet-Massin et al., 2012), for each of the algorithms we ran 10 pseudo-absence replicates with the same number of pseudo-absences as number of presences (n=1552). The models were fitted with 10 different random presence sets for each pseudo-absence run. Occurrence records were randomly divided into two subsets containing data for calibration (70%) and evaluation (30%) of models.

The ability of the models to predict the currently observed distribution of species was evaluated using the area under the receiver-operating characteristic (ROC) curve, Cohen's Kappa statistic and true skill statistic (TSS). Due to the lack of independent and reliable fossil records from the LGM for this species, the accuracy measures were calculated with reference to the current potential distribution only. The contributions of the variables to the models were obtained with a permutation procedure. We also performed ensemble forecasting (Thuiller et al., 2009) to generate final consensus models and to identify areas classified as suitable by the majority of the algorithms. Binary transformation was carried out using a threshold that maximized the true skill statistics (TSS) to generate the most accurate predictions (Jimenez-Valverde and Lobo, 2007).

RESULTS

With the 6 primers used, 412 unambiguously scoreable bands were generated for 200 investigated individuals in 37 populations (Tab. 1). Data quality tests indicated high repeatability across of the ISSR bands, above 97%. Fragment length varied between 150 and 1000 bp. The mean number of bands per individual *FA* ranged from 29.2 in population SL1 to 55.5 in population RO1. Shannon's diversity *I* ranged between 0.0323 in population SU1 and 0.1567 in RO1. The rarity index *DW* was 0.532 in population SM3 and 16.88 in population CR1 (Tab. 2). The number of individuals per population n was correlated with Shannon's diversity *I* (Person's r = 0.40), and the mean number of bands per population *FA* was positively correlated with Shannon's diversity *I* (Pearson's r = 0.58), and negatively correlated with the rarity index *DW* (Pearson's r = –0.46). The highest correlation was between the standard deviation of bands *FA(SD)* and the *DW* index, amounting to r = 0.66. All these correlations were statistically significant at $P < 0.05$.

Assessment of modeling success using three different tests (ROC, Cohen's Kappa, TSS) consistently indicated that the RF model was the best performer among the algorithms used (Supplementary material, Fig. S1a,b,c). The models using the other five algorithms achieved lower values but were also satisfactory. The most common method of model evaluation is the threshold-independent area under the curve (AUC) of the receiver operating characteristic (ROC) plot. The AUC scores obtained for individual models ranged from 0.88 to 0.94. According to the criteria described by Swets (1988), AUC values between 0.8 and 0.9 indicate good models and values between 0.9 and 1 indicate excellent models.

The test of variable importance showed relatively high scores for temperature-related climate variables. In particular, mean temperature of coldest quarter (*temp_cq*) was identified as the most important variable that may limit the range of the species (Supplementary material, Fig. S1d). Precipitation-related variables proved of minor importance.

Most of the models identified four isolated and narrow zones of putative glacial refugia of the grass species during the LGM (Fig. 1d): the first in the Atlantic, the second in the Mediterranean-Apennine region, the third forming an isolated and scattered Illyrian refugium in the West Balkans, and the fourth refugium for *B. erectus* possibly existing in the Pontic area along the southern and eastern coastlines of the Black Sea.

STRUCTURE and Neighbor-net analyses showed three genetic groups in the *B. erectus* populations (Supplementary material, Tab. S1, Fig. 2a,b). The first, group E1 (DE1–DE4), is confined exclusively to Germany. The second, group W, is distributed mainly in Central Europe north and west of the Alps, in the Czech Republic, Slovakia, Poland, Hungary, Romania, Austria and France. Population SM2 is genetically heterogenous and consists of both genetic groups E1 and E (Fig. 2a,b). It formed a subset of the German populations, with eleven bands absent and with one private allele (Supplementary material, Tab. S2). The third, group E, is distributed mainly in Bohemia and Moravia (Czech Republic) the Sudetes, Hungary, France (FR7) and Germany (DE5). Two populations (AU3, PL2) were highly polymorphic genetically.

The first three axes in PCoA analysis of the ISSR data explained 7.57%, 5.54% and 4.62% of the variation (total 17.73%; Fig. 3). The ordination of the individuals along Axis 1 revealed the distinctness of the E1 group of DE1–DE4 populations from Germany. On axis 2, population SM2 (E/E1) is intermediate between E1 and the remaining groups along Axis 1, E/E1 is between groups E and W, and Sudetic-Moravian populations SM1, SM5–6 and SU1–2 take an isolated position within genetic group E, seen at left on the diagram (Fig. 3).

The mean number of ISSR bands in the German group E1 was significantly lower than in the remaining groups, as was its rarity index *DW*. In the geographical division of the population into western and eastern populations, the mean number of ISSR bands per individual *FA* and the rarity index *DW* are significantly higher in the eastern populations. The differentiation between southern and northern populations is also statistically supported for the *FA* index (Supplementary material, Fig. S2a,d).

In AMOVA, 57.51% of molecular variance was found within populations, and 42.49% between populations (Tab. 3). The division of the populations by geographical location: west versus east (LONG) and south versus north (LATID), discriminated according to the median values of longitudinal and latitudinal

TABLE 2. Population numbering, population code, region and location, number of individuals sampled (n), geographical coordinates and genetic diversity parameters of the 37 populations of *Bromus erectus* sampled in Central Europe

no.	Code	Region/Locality	n	Long.	Latit.	*%Pol*	*h*	*FA*	*FA(SD)*	*I*	*DW*
1	AU1	Oberösterreich/Haid	5	14°17'	48°21'	19.9	0.074	45.0	2.3	0.0780	0.734
2	AU2	Steiermark/Leoben	5	15°00'	47°34'	25.0	0.092	50.0	3.1	0.1060	0.838
3	AU3	Niederösterreich/Dopplerhutte	5	16°16'	48°30'	21.6	0.079	45.2	0.8	0.0895	0.824
4	AU4	Oberösterreich/Ettenau	5	12°79'	48°08'	21.1	0.077	41.2	3.4	0.0903	1.585
5	CR1	Zadarska Zupania/Mali Losinj	4	14°28'	44°31'	21.1	0.086	49.5	1.9	0.0860	16.880
6	CZ1	Středočeský kraj/Libochovicky	2	14°24'	50°18'	18.4	0.095	50.5	10.6	0.0749	5.427
7	DE1	Regierungsbezirk Stuttgar/Obersontheim	10	9°83'	49°07'	21.6	0.063	37.9	3.7	0.0818	1.378
8	DE2	Mittelfranken/Detwang	10	10°16'	49°39'	22.3	0.063	35.6	2.4	0.0815	0.714
9	DE3	Oberfranken/Altendorf	10	11°01'	49°80'	20.6	0.058	38.5	3.2	0.0694	1.037
10	DE4	Thüringen/Arnstadt	6	10°94'	50°82'	17.7	0.061	35.5	2.9	0.0716	1.225
11	DE5	Regierungsbezirk Stuttgart/Gaildorf	5	9°77'	49°00'	22.1	0.080	41.6	4.5	0.0977	0.908
12	FR1	Rhône-Alpes/La Bathie	4	5°82'	45°06'	19.7	0.080	44.0	7.5	0.0794	0.929
13	FR2	Beaujolais/Balanod	5	5°36'	46°46'	25.7	0.087	43.4	2.3	0.1129	0.814
14	FR3	Berry/Villequiers	4	2°80'	47°06'	19.4	0.075	43.8	3.9	0.0751	1.064
15	FR4	Région Centre/Averdon	5	1°29'	47°70'	25.0	0.093	51.0	1.7	0.1079	1.027
16	FR5	Région Centre/Vailly	5	3°12'	47°46'	27.2	0.097	48.0	6.6	0.1304	1.022
17	FR6	Beaujolais/Arcy-sur-Cure	5	3°75'	47°60'	23.8	0.080	42.0	5.0	0.0966	0.986
18	FR7	Cote-d'Or/Villeferry	5	4°52'	47°46'	26.9	0.098	4.09	5.5	0.1296	0.950
19	FR8	Sabaudia/Le Mollard	5	6°27'	45°32'	24.3	0.087	50.8	3.4	0.0936	2.565
20	HU1	Pilis Mts/Pilisszentlaszlo	6	18°98'	47°72'	21.6	0.077	43.5	3.0	0.0900	0.784
21	HU2	Mecsek Mts/Orfu	4	18°20'	46°12'	18.2	0.071	37.5	1.9	0.0762	1.070
22	IT1	Friuli-Venezia Giulia/Tauriano	4	12°84'	46°10'	18.2	0.070	39.3	3.0	0.0743	0.741
23	IT2	Emilia-Romagna/Pallazina	4	11°16	44°31	23.1	0.092	54.0	3.4	0.0910	1.005
24	IT3	Emilia-Romagna/Cantoniera Tugo	5	9°94'	44°47'	27.2	0.100	50.0	7.4	0.1296	0.908
25	IT4	Friuli-Venezia Giulia/Gabrovizza	5	13°56'	46°17'	21.6	0.074	38.0	6.1	0.0913	0.996
26	PL1	Pomorze Zachodnie/Sulechówek	5	15°45'	53°14'	22.8	0.088	49.2	3.5	0.0983	1.317
27	PL2	Wyżyna Małopolska/Pińczów	10	20°47'	50°47'	31.8	0.092	48.4	4.2	0.1243	3.970
28	RO1	Țara Bârsei/Arini	10	25°53'	45°87'	39.6	0.111	55.5	4.5	0.1567	7.905
29	SL1	Inner Carniola/Kozina	5	13°96'	45°60'	19.7	0.069	29.2	14.1	0.0827	1.416
30	SM1	Jihomoravsky kraj/Choryne	4	17°89'	49°50'	18.0	0.074	43.3	3.0	0.0749	0.889
31	SM2	Jihomoravsky kraj/Hradcovice	6	17°60'	49°06'	17.0	0.061	38.2	2.6	0.0673	1.274
32	SM3	Jihomoravsky kraj/Horni Nemcy	2	17°65'	48°92'	13.8	0.069	42.0	2.8	0.0440	0.532
33	SM4	Jihomoravsky kraj/Javornik	8	17°57'	48°87'	25.5	0.080	45.9	1.6	0.0994	0.600
34	SM5	Jihomoravsky kraj/Radejov	5	17°33'	48°86'	22.1	0.083	41.2	2.5	0.0980	0.633
35	SM6	Jihomoravsky kraj/Strilky	5	17°19'	49°14'	22.3	0.084	45.6	4.7	0.0962	0.838
36	SU1	Sudetes/Zołotno	2	16°38'	50°42'	12.1	0.058	39.0	1.4	0.0323	0.976
37	SU2	Sudetes/Zielone Ludowe	5	16°35'	50°40'	17.7	0.069	33.4	5.2	0.0778	1.134
		Mean (Median*)	5.4	13°96'*	48°08'*	22.05	0.080	43.7	4.0	0.0907	1.565

%Pol – percentage of polymorphic bands; *h* – Nei's index of gene diversity; *FA* – mean number of ISSR bands per individual; *FA (SD)* – standard deviation of *FA; I* – Shannon's index of diversity; *DW* – rarity index corresponding to frequency-down-weighted marker values per population; AU – Austria; CR – Croatia; CZ – Czech Republic; DE – Germany; FR – France; HU – Hungary; IT – Italy; PL – Poland; RO – Romania; SL – Slovenia; SM – Czech Republic (Moravia); SU – Sudetes Mts (Polish part).

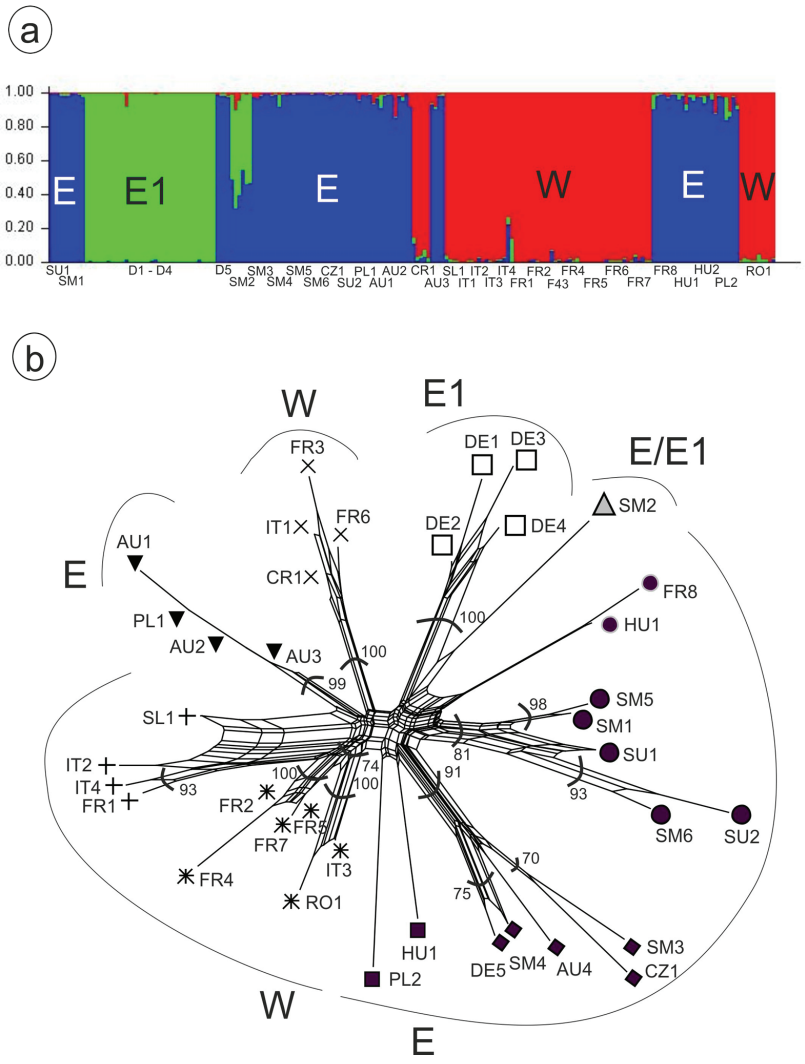

Fig. 2. (**a**) Bayesian admixture STRUCTURE analysis of *Bromus erectus* for K = 3. Three groups of genetic populations were distinguished: E, E1 and W, (**b**) Neighbor-net based on Nei-Li genetic distance among 37 *Bromus erectus* populations. Bootstrap values above 50% (1000 replicates) are presented at each node. The three groups W, E and E1 according to STRUCTURE analysis. AU – Austria; CZ – Czech Republic; DE – Germany; FR – France; HU – Hungary; IT – Italy; PL – Poland; RO – Romania; SL – Slovenia; SM – Czech Republic (Moravia); SU – Sudetes Mts (Polish part). Locality codes as in Table 2.

coordinates, was also significant at $P < 0.001$. The percentage of molecular variance explained was slightly lower for longitude (3.85%) than for latitude (4.32%) (Tab. 3).

General linear model regression showed trends of change in the number of ISSR bands in both S-N and W-E directions across Europe (Fig. 4, Tab. 4). The slope was negative for latitude LATID $\beta = -0.845223$ (d.f. = 32, $P < 0.10$) and positive for the exponent longitude LONG ^ 2 $\beta = 0.000643438$ ($P < 0.02$), and represented a multiplicative effect on the fitted value for each 1-unit increase in *FA*.

DISCUSSION

The genetic-geographic division of the grass species into two main groups, W and E, based on hypervariable ISSR markers, is in accordance with the syntaxonomic division of xerothermic grassland in Central Europe. Group E is linked with the Festucetalia valesiacea order which forms steppic and subcontinental xerothermic grasslands, with the area of the main geographical distribution in central Eastern Europe in Ukraine and in Central Europe in the Pannonian Basin, southern Moravia

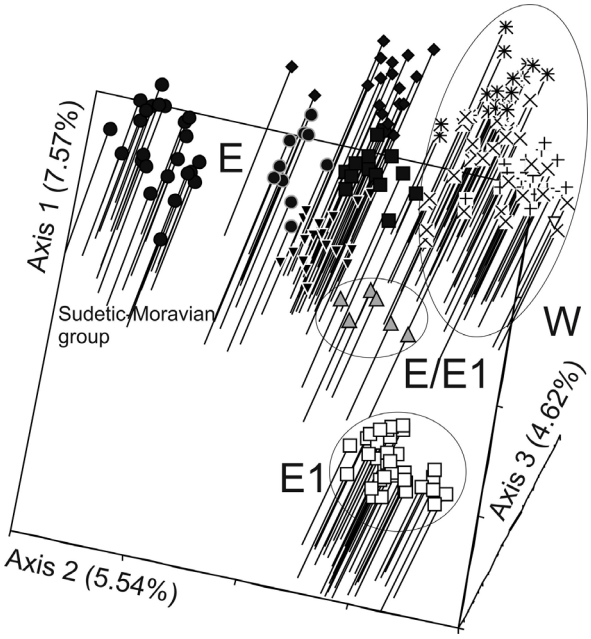

Fig. 3. Principal coordinate analysis (PCoA) of 200 individuals of *Bromus erectus*. Group codes and symbols as in Table 2 and Figure 2b.

TABLE 3. Analysis of molecular variance (AMOVA) for ISSR phenotypes of *Bromus erectus*. Regions were established according to median values of longitude (W and E) and latitude (N and S). *Fst* – fixation index

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*Fst*
A.	Among populations (total)	36	3373.41	13.91	42.49	0.42*
	Within populations	163	3068.96	18.83	57.51	
B.	E and W regions	1	184.12	1.25	3.85	0.44
	Among populations within regions	34	2516.19	13.08	40.47	
	Within populations	159#	2393.64	18.00	55.68	
C.	S and N regions	1	243.88	1.45	4.32	0.41
	Among populations within regions	35	3129.53	13.17	39.38	
	Within populations	163	3068.96	18.83	56.30	

*All P-values were <0.001, # – population AU4 removed from the analysis

and the eastern part of Lower Austria. The German group E1 could be regarded as a subset of the German populations DE1–DE4. The second genetic group W is linked with a syntaxonomic group of the Brometalia erecti order with a Subatlantic-Submediterranean distribution in France, Italy and Slovenia (Supplementary material, Fig. 3S). Exceptions were populations RO1, FR8, and partially DE5, which occurred as outliers. They could represent long-distance migrations or else might have their origin in unrecognized genetic centers.

The genetic division of *B. erectus*, supplemented by climate modeling of the LGM, points to the existence of two refugial areas of the species in Europe. The first is linked with the Atlantic-Mediterranean area and the second with the Pontic-Pannonian area. GLM regression analysis showed a decrease in the number of ISSR bands per population, 4% per degree of longitude along the N-S gradient. A decrease of the genetic diversity of a species is predicted in populations on the margin of a geographic distribution, where the founder effect and ecological bottlenecks play a distinct role, as for example in *Iris aphylla* at its range margin in the Polish lowlands (Wróblewska and Brzosko, 2006) or in *Froelichia floridana* in the southeastern United States (McCauley and Ballard 2002). Marginal or fragmented populations can be poor in number of alleles (bands) but otherwise highly specific, that is, with high values of the rarity index *DW*, as for exam-

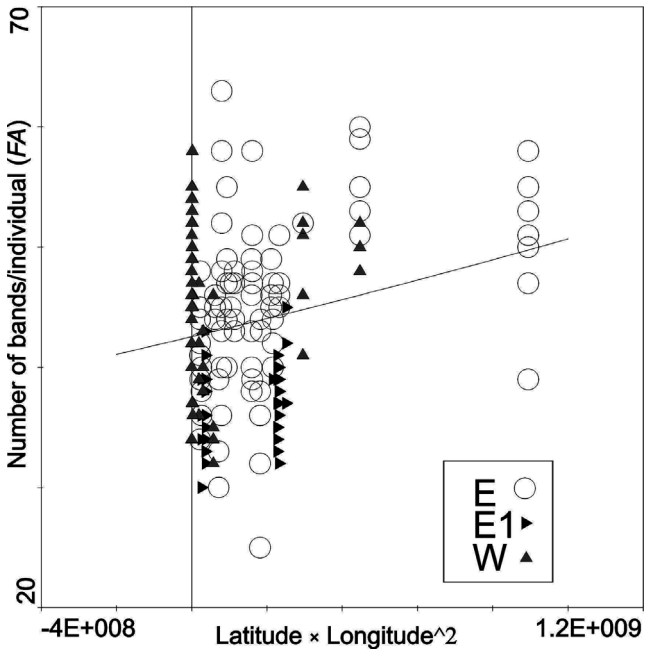

Fig. 4. General linear model (GLM) with a quadratic term (Tab. 4) to explain changes in mean number of ISSR bands *FA* along geographical coordinates; E, E1 and W genetic groups based on STRUCTURE analysis (see Fig. 2).

ple in *Aconitum bucovinense* in the Eastern Carpathians (Boroń et al., 2011) or *Melica transsilvanica* in Central Europe (Szczepaniak and Cieślak, 2009). In our present investigations such relict populations might be the ones in Austria (AU4), France (FR1), Hungary (HU1), Moravia (SM5) and the Sudetes (SU1). All of them have a high *DW* index and a low/moderate *FA* index. They could be remnants of genetically different populations (with private alleles) which then underwent a genetic bottleneck. They are scattered across Central Europe and their population genetic structure has been affected by fragmentation of xerothermic grasslands, shrinking their area on the European landscape (Karlik and Poschlod, 2011).

Interestingly, a similar pattern of genetic and geographic structure is displayed by the lycaenid butterfly *Polyommatus coridon*, a species characteristic of xerothermic grasslands of Southern and Central Europe. Genetic analyses of the species on the European scale revealed two glacial differentiation centers, western and eastern (Schmitt and Seitz, 2001). This bi-division is best explained by a long period of disjunction in two ice-age refugia, Adriatic-Mediterranean and Pontic-Mediterranean. They conform well to our projection of the potential distribution of *B. erectus* in the LGM climate scenario.

The weak though statistically significant decrease in the number of bands of *B. erectus* along the W-E gradient might be explained by differences

TABLE 4. Logistic regression model for *Bromus erectus* with a quadratic term to relate the mean number of ISSR bands per individual *FA* to geographic latitude (LATID) and longitude (LONG)

Model term	Coefficient value	SE	*T* value
LATID	-0.845223	0.46497	-1.8178
LONG	-0.0388963	0.0164288	-2.36756
LATID^2	0.00867284	0.00483943	1.79212
LONG^2	0.00158769	0.000643438	2.46751
Null deviance = 30.12, Deviance = 20.81, AIC = 6.454			
F = 4.13, P < 0.001			

in management practices between Western and Central Europe. Unlike in Western Europe, in countries of Central Europe the xerothermic grasslands were regularly mown and grazed up to the end of the last century. Abandonment of those management practices and the resulting successional changes have accelerated only in the last ten years or so. Extensive management, including moderate grazing and mowing, is believed to stabilize species-rich xerothermic grassland (Bąba, 2004), probably preserving the genetic diversity of the grass species.

A high Shannon's *I* index and a moderate *DW* index, pointing to a potential refugial pattern, were

found for southern populations from Austria (AU3), Italy, France, Slovenia, Romania and Hungary (HU2-HU3). The same status characterizes Central European populations SM1, SM4 and SM6. The Moravian Highlands (SE Czech Republic) have been postulated as a glacial refugium for many forest species, including *Bromus benekenii* (Sutkowska et al., 2014). The occurrence of the Central European genetic group E in population FR8 probably is the effect of recent migration, affected by unknown random events including human activity. Such processes were observed by Daneck et al. (2011) in studies of Central European populations of *Lonicera nigra* and by Hensen et al. (2010) in *Stipa capillata* in Germany, Switzerland and Slovakia.

According to our results, the genetically depleted German populations DE1–DE4 of the E1 group were mostly similar in their ISSR profile to the Moravian population SM2. It remains an open question whether they are of refugial or secondary character. Their significant depletion of genetic diversity could be the result of the ecological and genetic bottlenecks in the LGM (refugial hypothesis) or a founder effect in the Holocene (long-distance migration hypothesis). A detailed inspection of their ISSR pattern revealed that German populations formed a subset of the genetically rich Moravian population SM2. Thus, in spite of their genetic differentiation from the other populations they could be regarded as young secondary populations which probably originated in the Holocene from the genetic stock of the Bohemian-Moravian metapopulation. Such a scenario was adopted for another xerothermic species, *Eryngium campestre*, investigated across Germany (Bylebyl et al., 2008), and for the rare grass *Stipa capillata* (Hensen et al., 2010). Those authors found statistically significant differences in the species' gene diversity and the percentage of polymorphic bands between glaciated and unglaciated regions, and low genetic diversity due to a strong bottleneck as a result of the founder effect. High genetic diversity in the unglaciated region of the Rhine valley indicates a contact zone where lineages of different origin could meet. The highly polymorphic population PL2 may be a product of a melting pot where different migratory routes of grass species met. Pärtel et al. (2005) note, however, that for the majority of species that are endemic to the biome of temperate European grasslands, glacial survival in refugia far beyond Central Europe is unlikely. If so, such Central-European xerothermic plant species (e.g., *Anthylis vulneraria, Brachypodium pinnatum, Carlina acaulis, Gentianella pilosa, Potentilla alba*; Kotańska et al., 2000) may also have persisted through the LGM in situ.

Bromus erectus apparently has two centers of differentiation in Europe: in the Atlantic-Mediterranean and the Balkan-Pannonian. The bi-division is in conformity with the present syntaxonomic split of the Festuceto-Brometea class into the Brometalia erecti and Festucetalia valesiacae orders. The origin of the Central European populations probably is associated with postglacial migrations from the west and the south. Pointing to this is the decreasing number of ISSR bands from south to north and to a lesser extent from west to east. The latter gradient may also be affected by differences in management between Western and Central Europe, as grazing and mowing continued almost to recent times in Central Europe. The depleted genetic stock of the German populations seems to be of recent origin, very likely sourced in Moravia and Bohemia where xerothermic grass species probably survived the last LGM.

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SUPPLEMENTARY MATERIAL

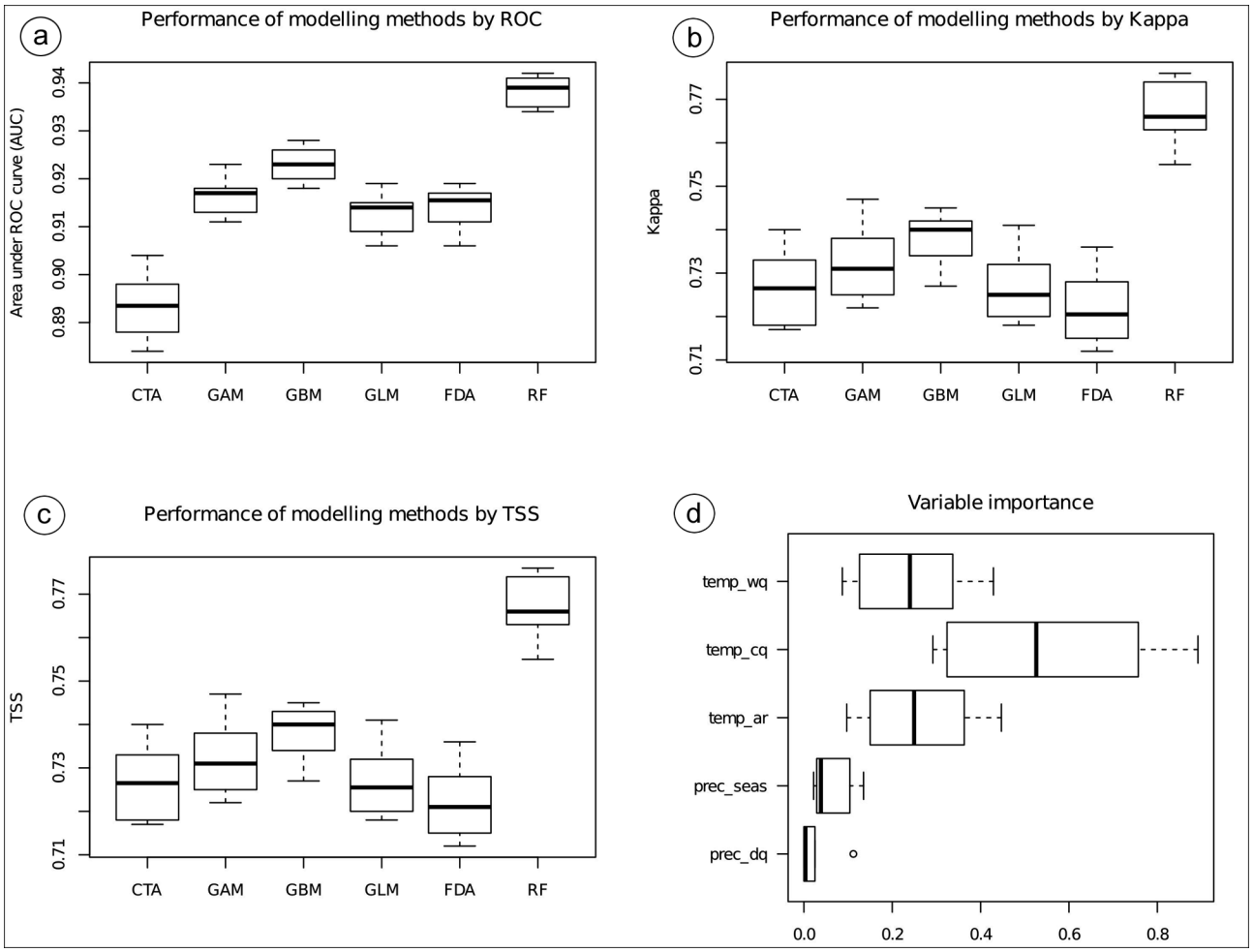

Fig. S1. Performance of modeling methods measured by (**a**) receiver operating characteristic (ROC), (**b**) Cohen's Kappa statistics, (**c**) true skill statistic (TSS) and (**d**) evaluation of variable importance.

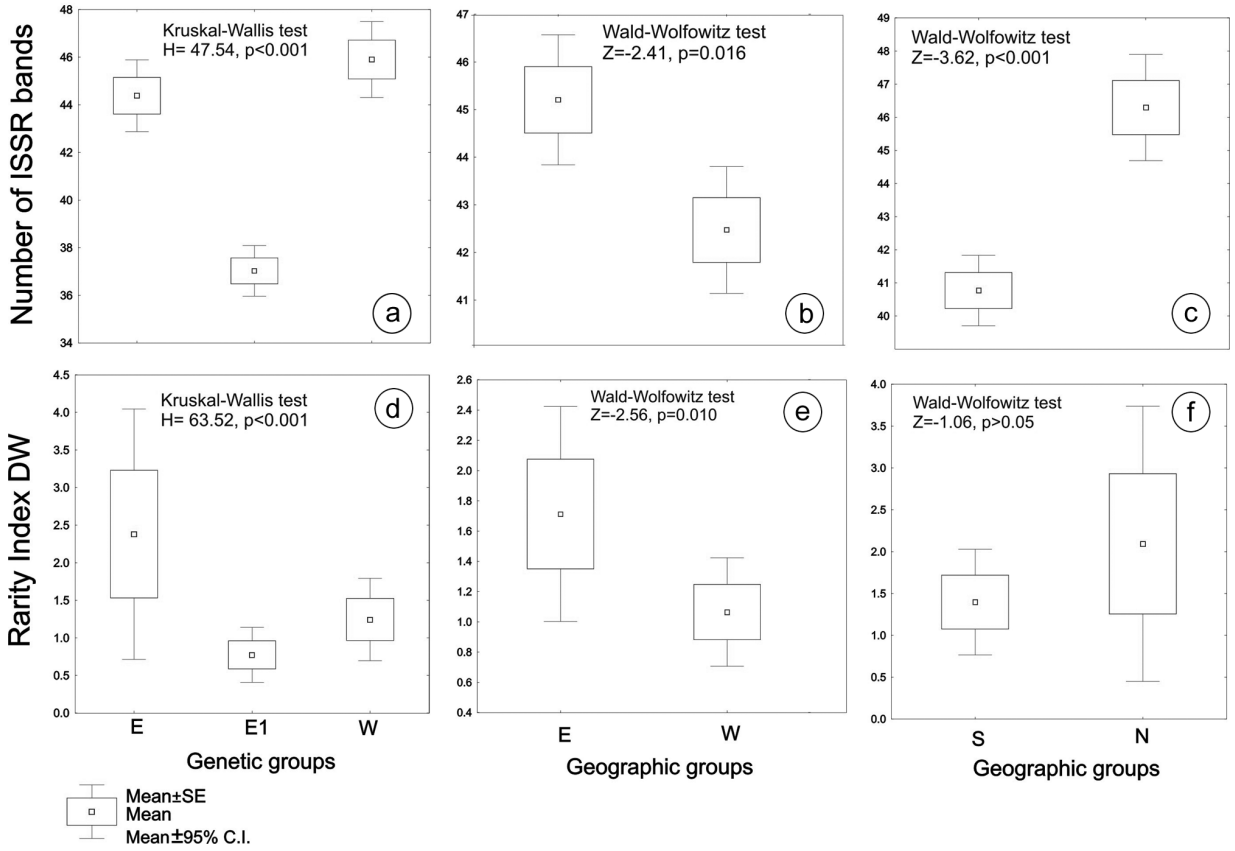

Fig. S2. Non-parametric statistical tests for differences in mean number of ISSR bands FA (**a, b, c**) and rarity index DW (**d, e, f**) and among three genetic groups (**a, d**) and four geographical groups (east and west **b–e**; south and north **c–f**) of *Bromus erectus* in Europe. Genetic groups according to STRUCTURE analysis (Figs. 1, 2); geographical groups according to median value of longitude and latitude coordinates (Tab. 2).

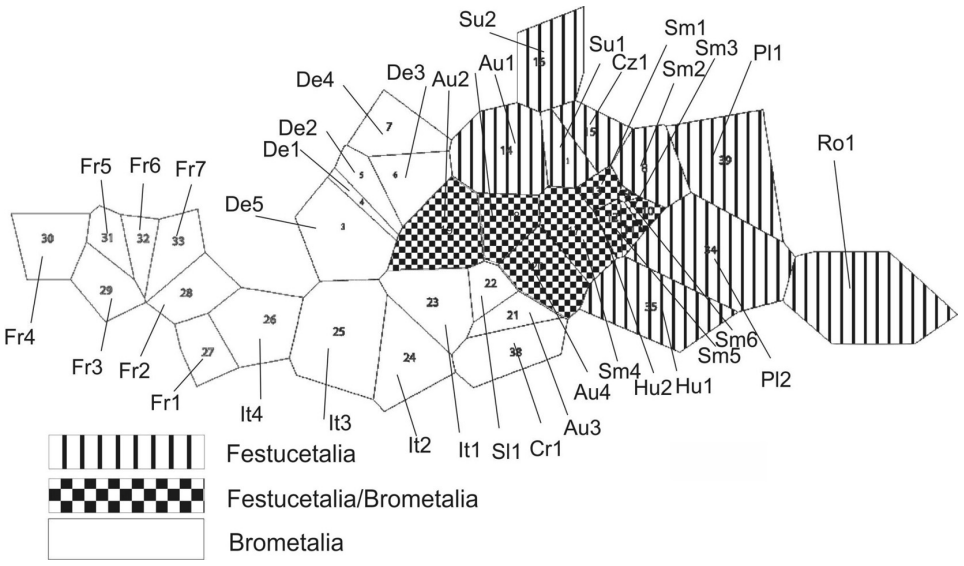

Fig. S3. Syntaxonomic units of the Festuco-Brometea class (Festucetalia and Brometalia orders) across Europe mapped on Voronoï's (1908) diagram based on the geographical coordinates of the studied localities (see Tab. 2). Au – Austria, Cr – Croatia, De – Germany, Fr – France, Hu – Hungary, It – Italy, Pl – Poland, Ro – Romania, Sl – Slovenia, Sm – Czech Republic (Moravia), Su – Sudetes Mts (Polish part).

TABLE S1. Results of STRUCTURE analysis using Structure-sum R-script ((Ehrich, 2006)

K	no. of runs	mean LnPData	standard dev.	L'(K)	L''(K)	ΔK
2	10	-21358.07	132.987	NA	NA	NA
3	10	-20153.75	35.95	1204.32	378.35	10.524
4	10	-19327.78	72.519	825.97	181.08	2.497
5	10	-18682.89	94.092	644.89	30.02	0.319
6	10	-18068.02	191.446	614.87	14.93	0.078
7	10	-17438.22	156.00	629.8	202.21	1.296
8	10	-17010.63	194.281	427.59	1124.7	5.789
9	10	-17707.74	2703.915	-697.11	2333.99	0.863
10	10	-16070.86	236.492	1636.88	NA	NA

K	no. of runs	mean similarity coeff.	standard dev.
2	10	0.522286512303071	0.628380421465852
3	10	0.500737210902433	0.322465025829314
4	10	0.344764103129735	0.328602802901926
5	10	0.187147915111811	0.185643393270099
6	10	0.185646213139173	0.153238528228408
7	10	0.293647989699162	0.154599180466392
8	10	0.257417612832179	0.117335114384151
9	10	0.4169892782905	0.138171928060175
10	10	0.550893820035493	0.226771896746069

NA – not analyzed

TABLE S2. Block structure of ISSR bands of population SM2 (E) and DE1-D4 (E1) of *Bromus erectus* (see Tab. 2 and Figs. 2, 3). Populations E1 forms a subset of group E. ISSR band no. 123 occurs only in E

Population	9	9	9	9	9	9	4	5	5	6	4	5	6	4	4	4	6	5	7	6	6	6	5	4	4	6	5	7	4	4	5	4	7	6	5	7	5	6	5	7	6	7
ISSR	E	E	E	E	E	E	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1
55							■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
121								■	■		■	■		■				■					■		■		■		■	■	■	■	■		■		■		■				
145							■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
174							■	■	■	■	■		■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■
201							■		■		■	■	■	■			■	■		■	■		■	■	■	■				■		■									■	
245							■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■			■	■			■	■	■	
256							■	■		■		■	■		■	■				■			■	■		■	■			■	■		■			■				■	■		
260							■	■	■	■	■	■	■	■	■	■		■	■	■	■	■		■	■				■	■	■	■	■	■	■	■	■		■	■	■	■
353							■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
384							■	■	■		■	■		■	■	■		■	■					■	■			■	■	■	■	■	■	■	■		■	■			■	■		■
393								■			■			■	■			■							■	■			■	■	■	■	■		■			■			■		■
44	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
61	■	■	■	■	■	■		■	■	■		■	■				■	■	■	■		■	■			■		■			■		■	■	■	■	■	■	■	■	■	
64	■	■					■			■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
68		■		■		■	■	■		■	■	■	■	■	■		■		■	■	■	■	■		■	■	■		■	■	■		■	■	■		■		■		■	■	■
111	■	■	■	■	■	■		■	■	■			■				■	■	■	■	■	■	■		■	■	■			■	■		■	■		■	■	■	■	■	■	■
163	■		■	■		■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■		■	
184	■	■				■	■		■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■		■		■	■	■	■	■		■	■
191	■	■	■	■	■	■		■	■	■		■	■		■		■	■	■	■	■	■	■	■	■		■	■	■	■		■	■	■		■	■	■	■	■	■	■
205	■			■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
252	■	■	■	■	■	■	■	■		■		■	■	■			■		■	■	■	■		■	■	■	■				■		■					■			■	
269	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
123	■	■	■	■	■	■																																				